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Foxo3 is a PI3K-dependent molecular switch controlling the initiation of oocyte growth

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ABSTRACT

In mammals, oocytes are packaged into compact structures–primordial follicles–which remain inert for prolonged intervals until individual follicles resume growth via a process known as primordial follicle activation. Here we show that the phosphoinositide 3-kinase (PI3K) signalling pathway controls primordial follicle activation through the forkhead transcription factor Foxo3. Within oocytes, Foxo3 is regulated by nucleocytoplasmic shuttling. Foxo3 is imported into the nucleus during primordial follicle assembly, and is exported upon activation. Oocyte-specific ablation of Pten resulted in PI3K-induced Akt activation, Foxo3 hyperphosphorylation, and Foxo3 nuclear export, thereby triggering primordial follicle activation, defining the steps by which the PI3K pathway and Foxo3 control this process. Inducible ablation of *Pten* and *Foxo3* in adult oocytes using a new tool for genetic analysis of the germline, *Vasa-Cre*^{ERT2}, showed that this pathway functions throughout life. Thus, a principal physiologic role of the PI3K pathway is to control primordial follicle activation via Foxo3.

Introduction

Primordial follicles, each comprised of a single oocyte and a surrounding layer of somatic granulosa cells, are formed early in life and represent a finite resource that must be safeguarded and efficiently utilized. The depletion of primordial follicles culminates in reproductive senescence or menopause, leading to the acceleration of many age-associated changes in women, such as reduced bone density (Block, 1952; Lobo, 2007). Primordial follicle activation is the irreversible, metered process by which primordial follicles are continually recruited to initiate follicle maturation (McGee and Hsueh, 2000). This process begins immediately after follicle assembly within a few days of birth and is gonadotropin-independent (Mason et al., 1986; Peters et al., 1973). Follicle growth is irreversible, and follicles that have initiated growth either complete the process (culminating in ovulation), or undergo atresia at some stage of their maturation. The irreversibility of this process and the limited supply of primordial follicles further suggest that primordial follicle activation is likely to be tightly regulated. Yet, the molecular pathway(s) regulating primordial follicle activation remain ill-defined, limiting our ability to understand this fundamental aspect of ovarian development and function that influences both fertility and the menopause.

The Foxo family of forkhead transcription factors (Foxo1, Foxo3, Foxo4, and Foxo6) participates in diverse processes including cell proliferation, apoptosis, stress resistance, differentiation, and metabolism (Accili and Arden, 2004). Foxo1, Foxo3, and Foxo4 are highly expressed in most tissues, and share partially overlapping functions, whereas Foxo6 expression appears to be restricted to the brain. Consistent with their participation in diverse processes, the Foxos are regulated by a variety of mechanisms including phosphorylation, acetylation, and ubiquitination (van der Horst and Burgering, 2007). Recently, we showed that the forkhead transcription factor Foxo3 is a master regulator and suppressor of primordial follicle activation, the first such factor to be defined. In *Foxo3* knockout mice, primordial follicles are assembled normally (John et al., 2007) but then immediately undergo global activation, resulting in a distinctive syndrome of ovarian hyperplasia, follicle depletion, premature ovarian failure, and infertility (Castrillon et al., 2003; Hosaka et al., 2004). However, many basic questions regarding the regulation of Foxo3 and its role in the suppression of primordial follicle activation remain unanswered. For example, it is not known if Foxo3 is part of a mechanism that actively controls the decision to trigger oocyte growth, or if Foxo3 mutation promotes follicle activation via some other, less direct mode of action.

The phosphatidylinositol 3-kinase (PI3K)-Akt signalling pathway is an important regulator of cell proliferation and survival, widely studied because of its participation in cancer and other disease processes (Cully et al., 2006). More recently, PI3K pathway components including Pten have been implicated in stem cell maintenance and the regulation of organ size (Groszer et al., 2001; Yilmaz et al., 2006) suggesting that this pathway plays general, albeit incompletely understood roles in tissue maintenance. PI3K catalyzes the production of the phosphoinositide PI(3,4,5)P3 (PIP3) from PI(4,5)P2 in the

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plasma membrane, resulting in membrane recruitment, phosphorylation, and activation of Akt. Pten serves as a potent PI3K antagonist by removing the 3′ phosphate from PIP3 (Engelman et al., 2006), thereby inhibiting Akt. Activated, phosphorylated Akt in turn phosphorylates a wide range of direct intracellular targets containing a minimal Akt recognition motif, including Gsk3, Bad, Tsc2, and the Foxos, among many others; at least 20 bona fide Akt substrates have been identified (Brunet et al., 1999; Manning and Cantley, 2007). However, it has been difficult in most experimental systems to fully define the physiologically relevant substrates and their relative contributions in mediating the biological effects of PI3K–Akt signalling.

In this paper, we show that the PI3K–Akt pathway has a key role in the initiation of oocyte growth (and hence in the maintenance of oocytes) and acts via Foxo3. Oocyte-specific *Pten* ablation resulted in Akt hyperactivation, Foxo3 hyperphosphorylation, and Foxo3 nuclear export, culminating in global primordial follicle activation and premature ovarian failure. Surprisingly, oocyte-specific ablation of Pten and Foxo3 resulted in virtually identical phenotypes of global primordial follicle activation, arguing that Foxo3 is the primary, if not sole effector of PI3K–Akt signalling in this physiologic context. Pharmacologic inhibition of PI3K suppressed the *Pten* but not the *Foxo3* ovarian phenotype, further establishing that *Foxo3* lies downstream of *Pten*. Our results demonstrate that Foxo3 is the prime effector of the PI3K–Akt pathway in the context of primordial follicle activation and that, surprisingly, the only essential role of Pten within the oocyte is to regulate Foxo3.

Materials and methods

Mouse strains, breeding, and analysis

This study was approved by an Institutional Animal Care and Use Committee. All alleles were in an FVB/n background (backcrossed at least $n=6$ generations). Genotyping of the *Vasa-Cre*, *R26R*, *Pten*, and *Foxo3* alleles was performed on tail DNA using multiplexed PCR protocols as described (Castrillon et al., 2003; Gallardo et al., 2007; Li et al., 2002; Soriano, 1999); the protocol for *Vasa-Cre*^{ERT2} was the same as for *Vasa-Cre*. *Vasa-Cre/+* mice were bred to *Foxo3*$^{L/L}$ homozygotes to obtain *Vasa-Cre; Foxo3*$^{L/+}$ male progeny; males must be used for the second generation cross because of a potent maternal effect observed with *Vasa-Cre* (Gallardo et al., 2007). The *Vasa-Cre* transgene affects Cre-mediated recombination in germ cells and thus, *Vasa-Cre* carriers cannot transmit the *L* allele; e.g. *Vasa-Cre; Foxo3*$^{L/+}$ mice can only transmit the null (−) or wt (+) *Foxo3* alleles. *Vasa-Cre; Foxo3*$^{L/+}$ males were crossed to *Foxo3*$^{L/L}$ females to generate experimental *Vasa-Cre; Foxo3*$^{-/L}$ females and sibling controls. An analogous strategy was employed to generate *Vasa-Cre; Pten*$^{-/L}$ animals and sibling controls. *Pten* floxed (Li et al., 2002) and *R26R* mice (Soriano, 1999) were purchased from Jackson Laboratories. Ovaries from at least $n=3$ experimental and $n=3$ control animals were evaluated for each timepoint in all analyses.

Tissue processing, immunohistochemistry and immunofluorescence

Tissue sections from experimental and control samples were placed on the same slide to ensure identical processing; at least $n=3$ slides were evaluated for each antibody. For immunohistochemistry, tissues were fixed in 10% formalin 1–12 h, then processed and embedded in paraffin. 5 μm sections were deparaffinized in xylene, and hydrated in an ethanol series. Slides were subjected to antigen retrieval by boiling in 10 mM NaCitrate and cooled at RT×20 min. Antibodies and titers used were: Foxo1 1:100 (Santa Cruz # sc-11350), Foxo4 1:200 (Santa Cruz # sc-5221), Pten 1:100 (Cell Signalling # 9559), p-Akt (Ser473) 1:50 (Cell Signalling # 9271), Foxo3 1:200 (Santa Cruz # sc-11351), p-Foxo3 (Thr32) 1:200 (Upstate # 07-695), p-mTOR (Ser2448) (Cell Signalling # 2976), p-S6 Ribosomal protein (Ser235/236) (Cell Signalling # 4857) and p-4E-BP1 (Thr70) (Cell Signalling # 9455). The detection system was Immpress (Vector, Burlingame, CA).

For immunofluorescence, tissues were fixed in 4% paraformaldehyde overnight at 4° and embedded in OCT. Frozen sections (5 μm) were obtained and detection of PIP3 was performed as described (Kitamura et al., 2004) with Biotin-PIP3 (1:100) (Echelon Biosciences # Z-B345b) and streptavidin-alexa fluor 488 (1:200) (Invitrogen # S11223). Images were obtained on an Olympus BX51 microscope equipped for epifluorescence.

Wholemount X-gal staining was performed as described (Gallardo et al., 2007).

Organ culture and histomorphometry

Ovaries were photographed after explantation and at 4 and 8 days and the relative ovarian volume was approximated by the equation $V=(\text{long diameter}\times\text{short diameter}^2)$. Average oocyte diameter was determined on H and E tissue sections per the long diameter of oocytes with nuclei in the plane of section (at least $n>100$ such oocytes were analyzed per ovary). Ovaries were cultured (37°, 5% CO_2) on Transwell Permeable supports (Costar, catalog # 3413) in Waymouth's medium (Invitrogen catalog # 11220-035) supplemented with 10% fetal bovine serum, 0.23 mM Pyruvic acid, 3 mg/ml BSA, 1× ITS supplement (Invitrogen, catalog # 51300-044) and 1× Antibiotic–Antimycotic (Invitrogen catalog # 15240-062). PI3K inhibition was performed by addition of 25 μM Ly294002 (Cell Signalling Technologies, catalog # 9901) to the media.

Generation of VasaCre^{ERT2} *transgenic mice and tamoxifen administration*

To generate the pVasaCre-ERT2 construct used for transgenesis, the SV40 promoter fragment (SalI fragment) was removed from pCre-ERT2 (Feil et al., 1997) by SalI digestion/religation. A polylinker was then inserted by annealing oligos 5′-CTAGGTCGACGGCGCGCCG-CGGCCGCTTAATTAA-3′ and 5′-GATCAATTAATTCGCCGGCGCCG-CGCGGCAGCTG-3′ and cloning into the AvrII site. The *Vasa* promoter fragment from pVasaCre (Gallardo et al., 2007) was released with AscI and PacI and cloned into the AscI and PacI sites of the above polylinker. The resulting 11,666 bp plasmid was linearized with SalI, purified on an Elutip-D column (Schleicher and Schuell) and microinjected into FVB/n oocytes by standard protocols (Nagy et al., 2003) at the UT Southwestern Transgenic Core Facility. Lines with transgene integration were identified by Southern blot, and further validated by Northern analysis. Total RNA (10 μg) was prepared from adult tissues using Tripure reagent (Roche), electrophoresed on a 1% formaldehyde gel, transferred to Hybond N+, probed with Cre and reprobed with *GAPDH* as a loading control. One line with high expression in testis and ovary (but not any other somatic tissues) was selected for further analysis and validation by crossing with *R26R* (Soriano, 1999). This line was backcrossed to FVB/n for 6 generations.

Tamoxifen (Sigma, catalog # T5648) was resuspended at 100 mg/ml in 100% ethanol and further diluted with corn oil (Sigma, catalog # C8267) to a final concentration of 20 mg/ml. Intraperitoneal injections (2 mg tamoxifen/100 μl corn oil) were given qD for three consecutive days. Tissues were collected 24 h following the last injection.

To exclude the possibility that tamoxifen might affect follicle growth, wild-type animals ($n=6$) were subjected to tamoxifen treatment. There was no evidence of increased activation three weeks after treatment: there was no alteration in follicle counts including the ratio of primordial to growing follicles, and there was no increase in oocyte diameter or change in pregranulosa cell morphology (data not shown). To exclude the possibility that the *Vasa-Cre*^{ERT2} allele results in altered follicle growth dynamics, ovaries ($n=6$ *Vasa-Cre*^{ERT2} females) were similarly analyzed and found to be morphologically unaltered (data not shown).

Results

Foxo3 localizes to the oocyte and undergoes nucleocytoplasmic shuttling

To begin to understand the role of Foxo3 in the suppression of primordial follicle activation, the Foxo3 protein was localized within mouse ovary tissue sections. Although prior mRNA *in situ* hybridization studies revealed widespread *Foxo3* expression throughout the ovary (Castrillon et al., 2003; Richards et al., 2002), the protein itself is detectable only within oogonia and oocytes (Fig. 1A). The closely related forkhead transcription factors Foxo1 and Foxo4 are not similarly localized to the oocyte (Fig. S1), rationalizing the unique, nonredundant role of Foxo3 in oogenesis (Paik et al., 2007; Tothova et al., 2007). At post-natal day (PD) 1, Foxo3 protein was cytoplasmic within most oogonia (Fig. 1A). By PD3, however, the protein had partially translocated to the nucleus and was detectable in both cytoplasm and nucleus in the great majority of follicles (Fig. 1B). By PD14, Foxo3 had completely translocated to the nucleus (Figs. 1A, B).

Foxo3 undergoes a number of post-translational modifications that regulate its activity, subcellular localization, and stability including acetylation, ubiquitylation, and phosphorylation by the kinases Ikkβ, Sgk, Cdk2, Dyrk1, Jnk, Ampk and Akt (Greer et al., 2007a; Greer et al., 2007b; van der Horst and Burgering, 2007). We speculated that the observed developmental translocation of Foxo3 protein was mediated by phosphorylation. Consistent with this hypothesis, a phospho-specific antibody that detects Foxo3 phosphorylated at its Akt consensus phosphorylation site RPRSC*T* (Thr32) detected p-Foxo3 (Thr32) in oocytes at PD3 (Fig. 1C). Furthermore, p-Foxo3 (Thr32) localized exclusively to the cytoplasm, suggesting that phosphorylation of Foxo3 by Akt regulates its subcellular localization within oocytes (Fig. 1C).

The timing of Foxo3 cytoplasmic-to-nuclear import coincides with the formation of individualized primordial follicles, which is completed by PD3, implying that nuclear Foxo3 is essential for suppression of follicle activation. Consistent with this interpretation, and further suggesting that Foxo3 restricts primordial follicle activation throughout life (see below), Foxo3 retains its nuclear localization in adult primordial oocytes (Fig. 1D). In growing follicles in wild-type adults, Foxo3 protein underwent a reverse shift from the nucleus to the cytoplasm, and then appeared to be rapidly degraded by the secondary follicle stage (Fig. 1D). The biological basis of this Foxo3 protein degradation has not been explored, but may be the result of ubiquitylation (van der Horst and Burgering, 2007). A prior study found that enforced expression of a constitutively active form of Foxo3 in maturing oocytes disrupts follicle maturation (Liu et al., 2007), suggesting that Foxo3 degradation in growing follicles is physiologically significant.

Foxo3 functions specifically within oocytes to suppress primordial follicle activation

These results suggested that although the gene is broadly expressed, Foxo3 might function specifically within oocytes as a molecular switch to regulate primordial follicle activation. To confirm this genetically, we employed a conditional (floxed) *Foxo3*^{L} allele and a *Vasa-Cre* (a.k.a. *ddx4-Cre*) deletor strain we previously generated (Castrillon et al., 2003; Gallardo et al., 2007; Paik et al., 2007). *Vasa-Cre* results in Cre-mediated recombination in >95% of oocytes in *Rosa26* β-galactosidase reporter (*R26R*) mice (Soriano, 1999) by PD3, with no

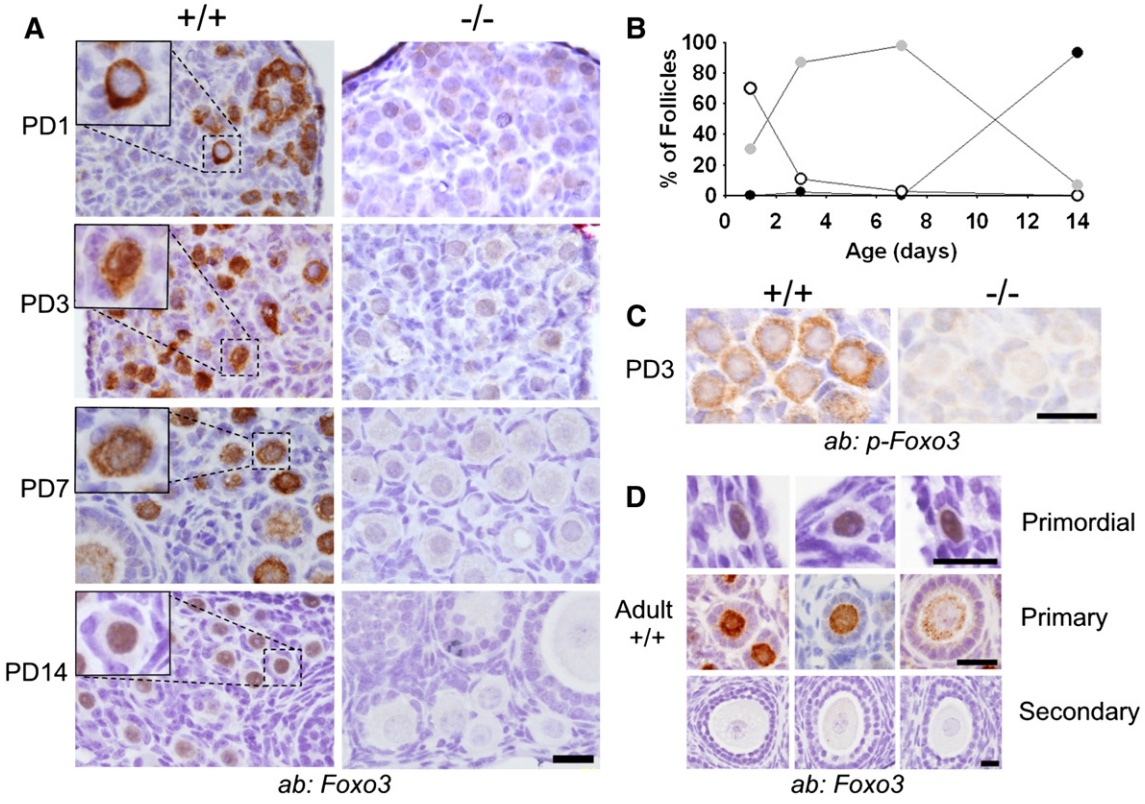

Fig. 1. Foxo3 nucleocytoplasmic shuttling during ovarian development and early follicle growth. (A) Immunolocalization of Foxo3 in *Foxo3*$^{+/+}$ and *Foxo3*$^{-/-}$ (negative control) ovaries, PD1–PD14. (B) Percent of follicles showing cytoplasmic (open circles), nuclear (black circles), or nuclear+cytoplasmic Foxo3 protein localization (gray circles) at PD1–14. (C) Immunodetection of p-Foxo3 (Thr32) at PD3. *Foxo3*$^{-/-}$ control confirms antibody specificity. (D) Nuclear to cytoplasmic translocation and degradation during early follicle growth, adult ovaries (6 weeks). Foxo3 localized to nucleus+cytoplasm in small primary follicles (2 left panels), but the protein was consistently cytoplasmic in larger primary follicles (right panel). Bars in all panels are 20 μm.

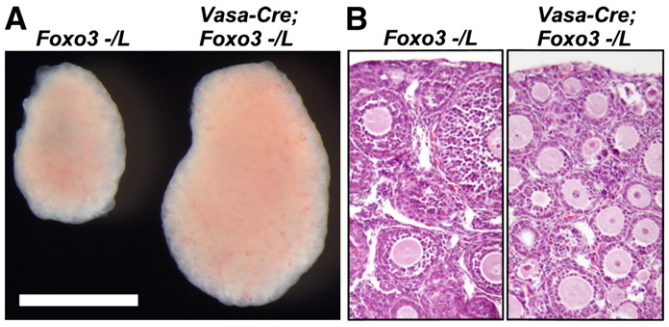

Fig. 2. Foxo3 functions within oocytes to suppress primordial follicle activation. (A) Ovarian hypertrophy in *Vasa-Cre; Foxo3*$^{-/L}$ female relative to sibling control. Bar = 1 mm. (B) Global follicle activation in *Vasa-Cre; Foxo3*$^{-/L}$ ovary; H and E-stained tissue section.

recombination in other ovarian cell types, and minimal recombination in other tissues (Gallardo et al., 2007). Ovaries from 3 week-old *Vasa-Cre; Foxo3*$^{-/L}$ females were dramatically enlarged relative to sibling controls (Fig. 2A), and histologic analysis demonstrated that this ovarian enlargement was due to global primordial follicle activation (Fig. 2B). These results constitute formal genetic proof that *Foxo3* functions specifically within oocytes to suppress primordial follicle activation, at least early in life.

Essential role of Pten and PI3K–Akt signalling in primordial follicle activation

To determine if Foxo3 in the context of primordial follicle activation is regulated primarily by PI3K–Akt signalling, we conditionally deleted the *Pten* gene in oocytes with *Vasa-Cre* and the previously described floxed allele *Pten*^{L} (Li et al., 2002). Consistent with prior results (Reddy et al., 2008), *Vasa-Cre*; *Pten*$^{-/L}$ ovaries, which have *Pten*-null oocytes, were dramatically enlarged by PD21; histologic analysis showed that this was due to widespread primordial follicle activation (Fig. 3). This confirms that Pten serves an essential physiologic role in the suppression of primordial follicle activation; in addition to Foxo3, it is the only other such factor identified (Castrillon et al., 2003). Surprisingly, in breeding tests with wild-type males, *Vasa-Cre*; *Pten*$^{-/L}$ females were initially fertile, although they exhibited a dramatic age-dependent decrease in fertility, having no more than two litters. Thus, within oocytes, Pten is specifically required for primordial follicle activation but not subsequent steps of oocyte growth, ovulation, or fertilization, a property it shares with Foxo3 (Castrillon et al., 2003; John et al., 2007). All progeny analyzed (n = 10) were heterozygous for the null *Pten* allele (data not shown), showing that the observed fertility was not due to "escaper" oocytes that failed to undergo Cre-mediated recombination. That the *Vasa-Cre Foxo3* and *Pten* mutant phenotypes have similar global activation phenotypes argues that Foxo3 is the chief physiologic target of the PI3K–Akt signalling pathway in the context of primordial follicle activation.

Oocyte Foxo3 is regulated by PI3K–Akt signalling

To confirm this hypothesis, we analyzed the status and subcellular localization of pathway components within oocytes. In control ovaries, Pten was readily detectable in primordial oocytes, where it localized to both nucleus and cytoplasm, but was undetectable in *Vasa-Cre; Pten*$^{-/L}$ oocytes, as expected (Fig. 4). Pten loss resulted in a dramatic increase of membrane-associated PIP3 in oocytes at PD3, promoting Akt membrane recruitment and activation, as evidenced by increased membrane-associated p-Akt. This increased Akt activity led to Foxo3 hyperphosphorylation and nuclear export (Fig. 4). We observed no evidence of mTOR pathway activation (by analyzing p-mTOR, p-S6K, and p-4EBP) in PD3 *Vasa-Cre; Pten*$^{-/L}$ oocytes (data not

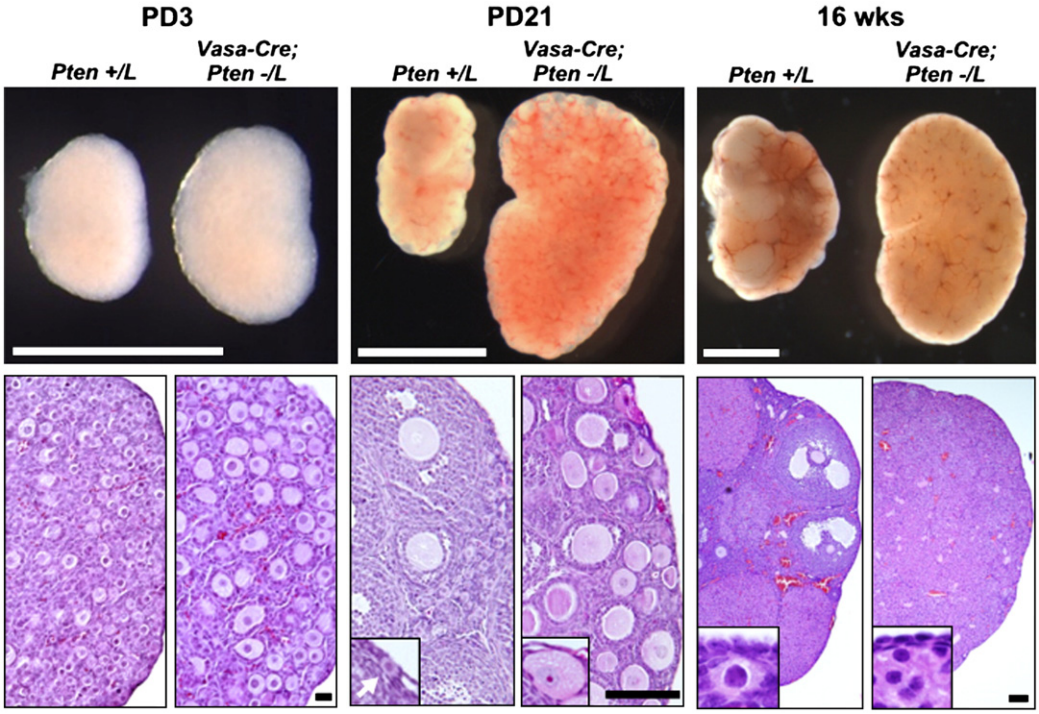

Fig. 3. Pten maintains egg supply and prevents ovarian failure. Upper panels: *Vasa-Cre; Pten*$^{-/L}$ and control ovaries at PD3, PD21, and 16 weeks; size bars = 1 mm. Lower panels, H and E-stained sections; size bars = (left to right) 20 μm, 100 μm, and 100 μm. At 16 weeks, controls have numerous primordial follicles whereas *Vasa-Cre; Pten*$^{-/L}$ ovaries are devoid of follicles, signifying complete ovarian failure. PD21 insets show primordial follicle in wild-type (arrow) and absence of primordial follicles in *Vasa-Cre; Pten*$^{-/L}$ ovary. 16 wk insets show normal primordial follicles in control ovary and stromal overgrowth/complete absence of follicles in *Vasa-Cre; Pten*$^{-/L}$ ovary. PD21 and 16 wk insets are not at same magnification.

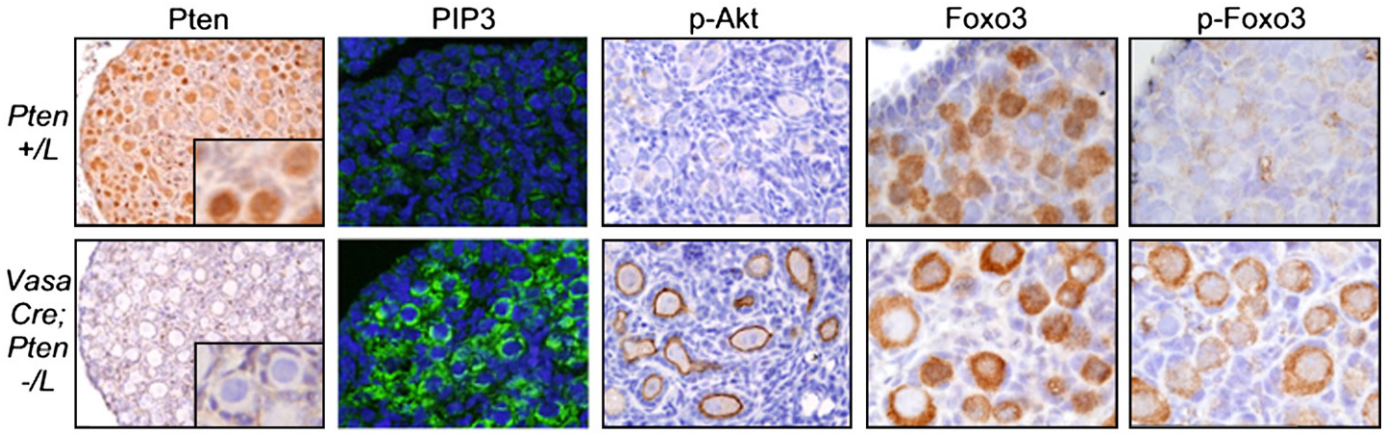

Fig. 4. Pten regulates primordial follicle activation via PI3K and Foxo3. *Pten* ablation leads to increased PIP3, Akt activation, Foxo3 hyperphosphorylation, and nuclear export. Panels show Pten, PIP3, p-Akt (Ser473), Foxo3 and p-Foxo3 (Thr32) in PD3 ovaries counterstained with hematoxylin or DAPI (PIP3).

shown). Thus, although the mTOR pathway normally becomes activated at much later stages of follicle growth (John et al., 2007), it does not appear to be a part of the mechanism that triggers follicle growth, further underscoring the role of Foxo3 as the prime effector of PI3K–Akt signalling in primordial follicle activation.

Pharmacologic inhibition studies confirm pathway linearity and that Foxo3 is downstream of Pten

To confirm that primordial follicle activation is regulated via the linear pathway (Pten–PI3K–Akt–Foxo3) suggested by these observations, ovaries were explanted at birth and cultured for 8 days in the presence of the PI3K inhibitor Ly294002. Untreated *Vasa-Cre; Pten*$^{-/L}$ and *Vasa-Cre; Foxo3*$^{-/L}$ ovaries grew more rapidly than controls. Ly294002 suppressed growth of *Vasa-Cre; Pten*$^{-/L}$ but not *Vasa-Cre; Foxo3*$^{-/L}$ ovaries, and measurements of average oocyte diameter, a more direct indicator of primordial follicle activation (Fig. 5) also showed that Ly294002 had a significant effect in *Vasa-Cre; Pten*$^{-/L}$ but not *Vasa-Cre; Foxo3*$^{-/L}$ ovaries ($p = 2.6 \times 10 \times 10^{-7}$ vs. 0.26, Student's *T* Test). Although it is possible that Ly294002 may have inhibited additional PI3K family members, these results demonstrate that the Pten (but not Foxo3) ovarian phenotype is PI3K-dependent, consistent with the above linear, ordered pathway where Pten regulates oocyte growth via P13K, PIP3, Akt, and Foxo3. Furthermore, these findings and the equivalence of the Pten and Foxo3 ovarian phenotypes including their initial fertility strongly argue that the only essential physiologic role of Pten within oocytes is to forestall primordial follicle depletion by maintaining Foxo3 within the nucleus.

Generation and validation of VasaCre^{ERT2}*, a novel tool for inducible gene ablation within the mouse germline*

The above genetic analyses utilized a *Vasa-Cre* transgene that is active starting at around embryonic day 15, and results in near-total Cre-mediated recombination by PD3 (Gallardo et al., 2007). Our findings were thus consistent with two alternate models. In the first model, PI3K–Akt signalling acts during this narrow developmental window (E15-PD3) to endow primordial follicles with some factor(s) that suppress follicle activation later in life, or are required only prior to PD3. Alternatively, the PI3K–Akt pathway might function continually throughout life to balance primordial follicle activation and preservation. To formally distinguish between these two models, an inducible, germ cell-specific *Cre*^{ERT2} transgene was generated by placing a *Cre*^{ERT2} cDNA under the control of the murine *Vasa* (*Ddx4*) promoter (*Vasa-Cre*^{ERT2}). The Cre^{ERT2} fusion protein is inactive until tamoxifen is administered (Feil et al., 1997). Tamoxifen treatment of *Vasa-Cre*^{ERT2}*; R26R* female mice at 6 weeks of age resulted in efficient (90–95%)

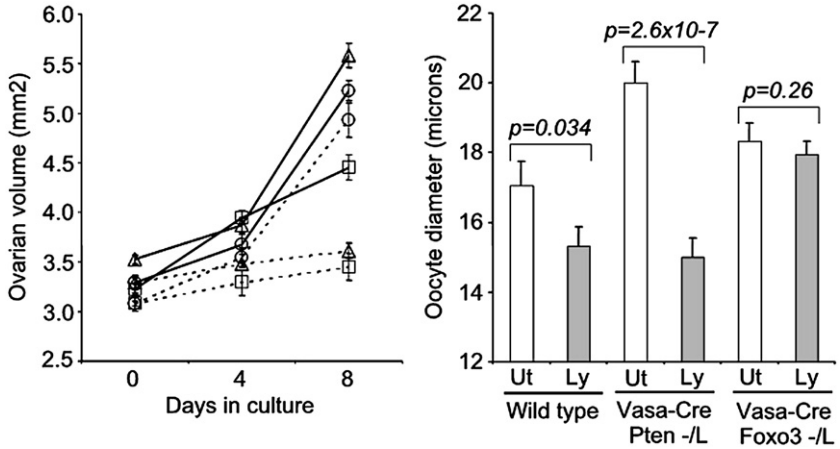

Fig. 5. Pharmacologic inhibition shows that PI3K activity is required for *Pten* but not *Foxo3* ovarian phenotype. Ovaries were explanted at birth and cultured for 8 days. Solid lines, untreated; dashed bars, 25 μM Ly294002. Squares = wild-type, triangles = *Vasa-Cre; Pten*$^{-/L}$; circles = *Vasa-Cre; Foxo3*$^{-/L}$. Graph on right shows oocyte diameters at the end of 8 day culture period. Ut = untreated, Ly = 25 μM LY294002. Note: The small apparent difference in untreated *Pten*$^{-/L}$ vs. *Foxo3*$^{-/L}$ oocytes is not significant ($p = 0.40$). Error bars = s. e. m.; *p* values calculated by Student's *T* Test.

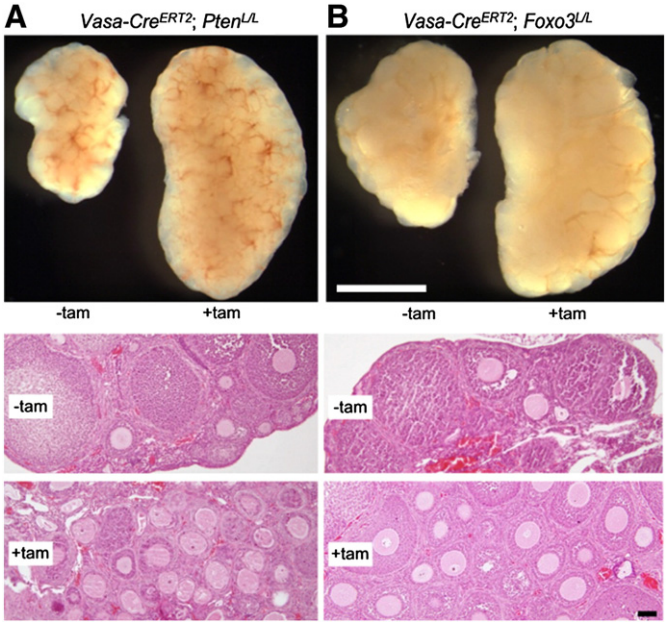

Fig. 6. Ablation of *Pten* and *Foxo3* in tamoxifen-treated adult *Vasa-Cre*^{ERT2} females results in global primordial follicle activation. (A) *Pten* (B) *Foxo3*. Bar = 1 mm. For both experiments, females were treated with tamoxifen and ovaries were harvested 3 weeks later; controls were untreated siblings of the same genotype. Ablation of *Pten* and *Foxo3* resulted in identical ovarian hypertrophy phenotypes; histological examination (H and E) confirmed that ovarian hypertrophy was due to global primordial follicle activation. Size bar = 50 μm. All corresponding panels in panels A and B are at the same magnification.

oocyte-specific recombination; there was no evidence of recombination prior to tamoxifen treatment (Fig. S2; see also Experimental procedures for additional details and controls). This efficiency was similar to that observed in the male germline at 3–6 weeks of age (Fig. S2, B–D). Incidentally, since production of active β-galactosidase requires 1) tamoxifen-induced transport of Cre from the cytoplasm to the nucleus, 2) homologous recombination of loxP sites, 3) transcription, mRNA transport, protein translation and transport, this experiment reveals that primordial follicles are normally engaged in a surprisingly high degree of metabolic activity and turnover of cellular components.

The Pten–PI3K–Akt–Foxo3 pathway that regulates primordial follicle activation functions throughout life

Vasa-Cre^{ERT2}*; Foxo3*$^{L/L}$ and *Vasa-Cre*^{ERT2}*; Pten*$^{L/L}$ females were generated by standard genetic crosses and treated at 6 weeks of age with tamoxifen. Ovaries were harvested following a three week interval to permit follicle growth and scoring of follicle growth phenotypes. Ablation of both *Pten* and *Foxo3* in adult oocytes resulted in identical phenotypes of ovarian hypertrophy; histologic analyses confirmed global follicle activation (Fig. 6). Similar results were obtained at 3, 9, and 12 weeks of age (not shown). Thus, this pathway functions throughout life to regulate the utilization of the egg supply through Foxo3.

Discussion

Primordial follicle activation appears to be a stochastic process, but the need to ration a finite supply of primordial follicles throughout reproductive life suggests that this process is tightly regulated. We speculate that activation is influenced by positive and negative feedback loops, perhaps mediated by secreted, diffusible factors. Although such factors have not been definitively identified (Skinner, 2005), our findings substantially advance our understanding of ovarian biology by demonstrating that such communication is integrated by Foxo3 and the PI3K signalling pathway acting within the oocyte itself (see Fig. 7 for model of Foxo3 function within oocytes).

Foxo1, Foxo3, and Foxo4 function similarly, are broadly expressed, and share partially redundant and overlapping functions, whereas Foxo6 is regulated differently and lacks a conserved Akt motif (van der Heide et al., 2005). In genetic analyses of Foxo1, Foxo3, and Foxo4 in mice, a prominent tumor suppressor phenotype was not apparent in single gene knockouts, whereas post-natal inactivation of all three loci resulted in lymphomas and endothelial cell neoplasms (Paik et al., 2007). Inactivation of two of three *Foxo* genes gave rise to intermediate phenotypes, clearly demonstrating that the Foxos can compensate for one another, at least in some cell types. Given this potential for genetic redundancy, it was initially surprising that Foxo3 has a unique, nonredundant role in the suppression of primordial follicle activation. This may now be explained by our finding that Foxo3 appears to be the predominant Foxo protein in oocytes. It is also notable that at the protein level, Foxo3 is readily detectable only

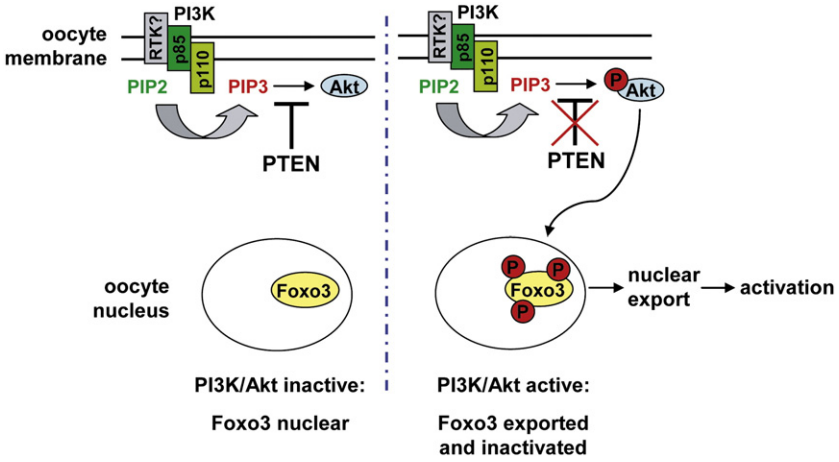

Fig. 7. Model of Foxo3 regulation by PI3K–Akt signalling pathway within oocytes in the control of primordial follicle activation. In resting state, absence of a presumptive ligand engaging receptor tyrosine kinase (RTK) complex or other cell surface receptor is associated with diminished Akt signalling in primordial oocytes. Foxo3 is unphosphorylated and thus localized to the nucleus, where Foxo3 acts to suppress primordial follicle activation. Binding of presumptive ligand activates PI3K (consisting of p85 regulatory and p110 catalytic subunits), which phosphorylates the 3′OH group of PI[4,5]P2 (PIP2) to form PI[3,4,5]P3 (PIP3) at the oocyte membrane. Pten acts as a potent negative regulator of this pathway by dephosphorylating PIP3. Pten inactivation results in accumulation of PIP3 at the oocyte membrane and hyperactivation of Akt, resulting in Foxo3 phosphorylation and nuclear export, thereby triggering primordial follicle activation. In this model, Pten inactivation is functionally equivalent to the binding of presumptative ligand at a cell surface receptor.

within oocytes, whereas at the mRNA level, Foxo3 is much more broadly expressed within the ovary (Castrillon et al., 2003; Richards et al., 2002). This observation suggests that post-transcriptional mechanisms regulating Foxo protein stability are highly significant in vivo. Consistent with this idea, Foxo protein stability has been found to be regulated by ubiquitylation and proteosomal targeting in a wide range of experimental model systems and cell types (Huang and Tindall, 2007; Plas and Thompson, 2003; Schisler et al., 2007).

We discovered that within oocytes, Foxo3 is phosphorylated at an Akt site and that the phosphorylation status of this site tightly correlates with subcellular localization (cytoplasmic vs. nuclear) in vivo, arguing that Akt is the prime regulator of Foxo3 in the context of primordial follicle activation. Concordantly, oocyte ablation of Pten led to Akt hyperactivation, Foxo3 hyperphosphorylation, and export of Foxo3 from the nucleus to the cytoplasm, resulting in follicle activation. It is also possible that additional kinases or other types of post-translational modification contribute to the regulation of Foxo3 within oocytes, serving as additional layers of regulation, but our findings argue that such modifications are not the principal mechanism by which Foxo3 is regulated as a switch controlling primordial follicle activation.

The mTOR protein participates in two distinct complexes, mTORC1 and mTORC2, with different biological functions and specificities (Guertin and Sabatini, 2007). Our finding that oocyte Akt is phosphorylated at S473 argues that mTORC2 is active (or becomes activated) in primordial oocytes, since mTORC2 has recently been shown to be primary kinase regulating Akt via phosphorylation at this site. On the other hand, we found no evidence of increased phosphorylation of mTORC1 targets during follicle activation. Since at least some of these targets, including p-S6K (John et al., 2007) and p-4EBP (unpublished data) become hyperphosphorylated in more advanced follicles, mTORC1 activation may be important in promoting increased protein translation during oocyte growth, but not during activation per se.

Along these lines, it is notable that *Pten* and *Foxo3* are genetically equivalent with regard to primordial follicle activation. Conditional deletion of both genes within oocytes using the *Vasa-Cre* transgene led to nearly identical phenotypes of global follicle activation. An even more striking demonstration of this genetic equivalence is that *Vasa-Cre; Pten*$^{L/L}$ and *Vasa-Cre; Foxo3*$^{L/L}$ females (as well as *Foxo3*$^{-/-}$ females) are initially fertile (Castrillon et al., 2003). Thus, neither Foxo3 nor Pten are essential for subsequent steps of follicle maturation, ovulation, fertilization, etc. These observations are further evidence that this pathway proceeds in a linear manner with Foxo3 downstream of Pten and PI3K/Akt, and that Foxo3 is the principal, if not sole output of this pathway. While the Foxos are known to be important effectors of PI3K/Akt signalling, we find it somewhat surprising that a single target, Foxo3, has such an overriding role as an effector of this pathway within oocytes, given the diversity of potential Akt substrates. It is also rather surprising that Pten inactivation and resultant Akt hyperactivation within oocytes does not disrupt subsequent steps of follicle maturation, ovulation, etc. Although it is likely that PI3K–Akt signalling participates in other aspects of oogenesis and follicle maturation, our findings demonstrate that this signalling pathway has evolved to serve a particularly critical role in primordial follicle activation.

Our investigations with *Vasa-Cre*^{ERT2} establish the feasibility of conditional gene targeting in primordial oocytes. This study represents the first example of conditional genetic inactivation in primordial oocytes in adult animals, and thus *Vasa-Cre*^{ERT2} should prove useful for a broad range of studies of oocyte activation, survival, and aging, as well as conditional genetic analysis of the male germ line (Fig. S2). It is also remarkable that the efficiency of recombination was so high (90–95% of primordial oocytes), at least up to 6 weeks of age. Primordial follicles are mitotically inactive (Hirshfield, 1991), and prior radiolabelling studies suggested that primordial oocytes are also metabolically quiescent (Bakken and McClanahan, 1978; Hirshfield, 1991; Moore and Lintern-Moore, 1974; Moore et al., 1974; Roversi and Silvestrini, 1963). However, these prior studies employed methods of limited sensitivity. Since production of active β-galactosidase requires 1) tamoxifen-induced transport of Cre from the cytoplasm to the nucleus, 2) homologous recombination of loxP sites, and 3) ATP-intensive processes including transcription, mRNA transport, and protein translation, the high efficiency of Cre-mediated recombination argues that primordial follicles are normally engaged in a surprisingly high degree of metabolic activity and turnover of cellular components. In these respects, our observations argue that primordial oocytes resemble neurons, a finding with intriguing implications for the age-associated oocyte loss that leads to the menopause. We speculate that oocytes may be subject to some of the same aging mechanisms that contribute to age-associated neurodegeneration, including oxidative stress and the accumulation of damaged or malfolded proteins (Mattson and Magnus, 2006).

Lastly, our findings, including the demonstration that the pathway functions continually in adults and not just during a more limited stage of early development, further support the idea that defects in PI3K signalling may contribute to certain forms of female infertility due to primordial follicle depletion, such as premature ovarian failure and primary amenorrhea. These findings also raise the possibility that pharmacologic agents acting upon the PI3K pathway, several of which are in clinical trials (Granville et al., 2006), may be useful in controlling follicle activation, treating infertility, or forestalling the menopause.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2008.06.017.

References

Accili, D., Arden, K.C., 2004. FoxOs at the crossroads of cellular metabolism, differentiation, and transformation. Cell 117, 421–426.
Bakken, A.H., McClanahan, M., 1978. Patterns of RNA synthesis in early meiotic prophase oocytes from fetal mouse ovaries. Chromosoma 67, 21–40.
Block, E., 1952. Quantitative morphological examinations of the follicular system in women. Acta Anatomica 14, 108–123.
Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J., Greenberg, M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. Cell 96, 857–868.
Castrillon, D.H., Miao, L., Kollipara, R., Horner, J.W., DePinho, R.A., 2003. Suppression of ovarian follicle activation in mice by the transcription factor Foxo3a. Science 301, 215–218.
Cully, M., You, H., Levine, A.J., Mak, T.W., 2006. Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis. Nat. Rev. Cancer 6, 184–192.
Engelman, J.A., Luo, J., Cantley, L.C., 2006. The evolution of phosphatidylinositol 3-kinases as regulators of growth and metabolism. Nat. Rev. Genet. 7, 606–619.
Feil, R., Wagner, J., Metzger, D., Chambon, P., 1997. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. Biochem. Biophys. Res. Commun. 237, 752–757.
Gallardo, T., Shirley, L., John, G.B., Castrillon, D.H., 2007. Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. Genesis 45, 413–417.
Granville, C.A., Memmott, R.M., Gills, J.J., Dennis, P.A., 2006. Handicapping the race to develop inhibitors of the phosphoinositide 3-kinase/Akt/mammalian target of rapamycin pathway. Clin. Cancer Res. 12, 679–689.
Greer, E.L., Dowlatshahi, D., Banko, M.R., Villen, J., Hoang, K., Blanchard, D., Gygi, S.P., Brunet, A., 2007a. An AMPK–FOXO pathway mediates longevity induced by a novel method of dietary restriction in *C. elegans*. Curr. Biol. 17, 1646–1656.
Greer, E.L., Oskoui, P.R., Banko, M.R., Maniar, J.M., Gygi, M.P., Gygi, S.P., Brunet, A., 2007b. The energy sensor AMP-activated protein kinase directly regulates the mammalian FOXO3 transcription factor. J. Biol. Chem. 282, 30107–30119.

Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X., Wu, H., 2001. Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo. Science 294, 2186–2189.

Hirshfield, A.N., 1991. Development of follicles in the mammalian ovary. Int. Rev. Cytol. 124, 43–101.

Hosaka, T., Biggs 3rd, W.H., Tieu, D., Boyer, A.D., Varki, N.M., Cavenee, W.K., Arden, K.C., 2004. Disruption of forkhead transcription factor (FOXO) family members in mice reveals their functional diversification. Proc. Natl. Acad. Sci. U. S. A. 101, 2975–2980.

Huang, H., Tindall, D.J., 2007. Dynamic FoxO transcription factors. J. Cell. Sci. 120, 2479–2487.

John, G.B., Shirley, L.J., Gallardo, T.D., Castrillon, D.H., 2007. Specificity of the requirement for Foxo3 in primordial follicle activation. Reproduction 133, 855–863.

Kitamura, T., Kitamura, Y., Nakae, J., Giordano, A., Cinti, S., Kahn, C.R., Efstratiadis, A., Accili, D., 2004. Mosaic analysis of insulin receptor function. J. Clin. Invest. 113, 209–219.

Li, G., Robinson, G.W., Lesche, R., Martinez-Diaz, H., Jiang, Z., Rozengurt, N., Wagner, K.U., Wu, D.C., Lane, T.F., Liu, X., Hennighausen, L., Wu, H., 2002. Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. Development 129, 4159–4170.

Liu, L., Rajareddy, S., Reddy, P., Du, C., Jagarlamudi, K., Shen, Y., Gunnarsson, D., Selstam, G., Boman, K., Liu, K., 2007. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development 134, 199–209.

Lobo, R.A., 2007. Menopause, In: Katz, V.L., Lentz, G.M., Lobo, R.A., Gershenshon, D.M. (Eds.), Comprehensive Gynecology, *5th edition*. Mosby Elsevier, Philadelphia.

Manning, B.D., Cantley, L.C., 2007. AKT/PKB signaling: navigating downstream. Cell 129, 1261–1274.

Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young 3rd, W.S., Phillips, H.S., Nikolics, K., Seeburg, P.H., 1986. A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. Science 234, 1366–1371.

Mattson, M.P., Magnus, T., 2006. Ageing and neuronal vulnerability. Nat. Rev. Neurosci. 7, 278–294.

McGee, E.A., Hsueh, A.J., 2000. Initial and cyclic recruitment of ovarian follicles. Endocr. Rev. 21, 200–214.

Moore, G.P., Lintern-Moore, S., 1974. A correlation between growth and RNA synthesis in the mouse oocyte. J. Reprod. Fertil. 39, 163–166.

Moore, G.P., Lintern-Moore, S., Peters, H., Faber, M., 1974. RNA synthesis in the mouse oocyte. J. Cell. Biol. 60, 416–422.

Nagy, A., Gertsenstein, M., Vintersten, K., Behringer, R., 2003. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., Jiang, S., Gilliland, D.G., Chin, L., Wong, W.H., Castrillon, D.H., DePinho, R.A., 2007. FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis. Cell 128, 309–323.

Peters, H., Byskov, A.G., Lintern-Moore, S., Faber, M., Andersen, M., 1973. The effect of gonadotrophin on follicle growth initiation in the neonatal mouse ovary. J. Reprod. Fertil. 35, 139–141.

Plas, D.R., Thompson, C.B., 2003. Akt activation promotes degradation of tuberin and FOXO3a via the proteasome. J. Biol. Chem. 278, 12361–12366.

Reddy, P., Liu, L., Adhikari, D., Jagarlamudi, K., Rajareddy, S., Shen, Y., Du, C., Tang, W., Hamalainen, T., Peng, S.L., Lan, Z.J., Cooney, A.J., Huhtaniemi, I., Liu, K., 2008. Oocyte-specific deletion of Pten causes premature activation of the primordial follicle pool. Science 319, 611–613.

Richards, J.S., Sharma, S.C., Falender, A.E., Lo, Y.H., 2002. Expression of FKHR, FKHRL1, and AFX genes in the rodent ovary: evidence for regulation by IGF-I, estrogen, and the gonadotropins. Mol. Endocrinol. 16, 580–599.

Roversi, G.D., Silvestrini, R., 1963. Study on the protein metabolism of the evolutionary ovarian follicle. Exp. Cell Res. 31, 484–489.

Schisler, J.C., Willis, M.S., Patterson, C., 2007. You spin me round: MafBx/Atrogin-1 feeds forward on FOXO transcription factors (like a record). Cell Cycle 7.

Skinner, M.K., 2005. Regulation of primordial follicle assembly and development. Hum. Reprod. Update 11, 461–471.

Soriano, P., 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat. Genet. 21, 70–71.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E. P., Lazo-Kallanian, S., Williams, I.R., Sears, C., Armstrong, S.A., Passegue, E., DePinho, R.A., Gilliland, D.G., 2007. FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress. Cell 128, 325–339.

van der Heide, L.P., Jacobs, F.M., Burbach, J.P., Hoekman, M.F., Smidt, M.P., 2005. FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling. Biochem. J. 391, 623–629.

van der Horst, A., Burgering, B.M., 2007. Stressing the role of FoxO proteins in lifespan and disease. Nat. Rev. Mol. Cell Biol. 8, 440–450.

Yilmaz, O.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H., Morrison, S.J., 2006. Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells. Nature 441, 475–482.